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A novel breast cancer specific gene BCSG1 was cloned by differential cDNA sequencing. BCSG1, a Alzheimer's disease (AD) amyloid like protein, is expressed in high abundance in breast cancer cDNA library but scarcely in normal breast cDNA library. Preliminary studies suggest that: 1) BCSG1 expression is a stage-specific in human breast: undetectable in normal or benign breast lesions, low level and partial expression in low grade ductal carcinoma *in situ* but extremely high level in advanced infiltrating breast cancer; 2) overexpression of BCSG1 in human breast cancer cells stimulates cell growth and invasion potentials *in vitro* and tumor growth in nude mice; 3) BCSG1 expression in breast cancer cells is dramatically suppressed by tumor growth inhibitor oncostatin M (OM), a cytokine predominantly produced by activated T cells and macrophages. This OM-induced transcriptional suppression of BCSG1 gene is associated with OM-mediated growth inhibition. The overall hypothesis to be evaluated is that overexpression of BCSG1 may correlate with clinical aggressiveness of breast cancer and lead to their abnormal growth and malignant progression. We will study the biological functions of BCSG1 on breast cancer growth and metastasis; to define its range of expression and begin to determine the mechanisms whereby BCSG1 expression is involved in breast cancer progression.

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I. ADJUSTMENT

Metastatic dissemination is the primary cause of death for most breast cancer patients. The research effort in PI's lab is to uncover the mechanisms whereby breast cancer undergoes malignant progression and becomes metastatic. The onset and progression of breast cancer are accompanied by multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (1). Our hypothesis is that many of these quantitative genetic changes manifest them as alterations in the cellular complement of novel transcribed mRNAs. Identification of these mRNAs could provide clinically useful information for patient management and prognosis while enhancing our understanding of breast cancer pathogenesis. Although pathological endpoints such as a tumor size, lymph node status and vascular invasion remain the most useful guides in prognosis and selecting treatment strategies for breast cancer (2), there is a need to further investigate the molecular mechanisms that determine the properties of an individual tumor, e.g., probability of metastasis. While numerous prognostic factors have now been identified, few have contributed to defining clinical response to therapy.

The current Career Development Award was initially awarded to study the novel 80kDa matrix degrading proteinase in breast cancer progression. However, as we stated in the last year's annual report, we have adjusted our proposed study to a complete new area based on the following reasons:

1. During the first two years of the grant, we have devoted many efforts on raising monoclonal antibodies to the 80kDa proteinase in an attempt to using these antibodies for purification and molecular cloning. Although we obtained the antiserum which can immunoprecipitate the 80kDa proteinase, no success has been met for development of monoclonal antibodies.

2.Dr. Robert Dickson, PI's former postdoctoral mentor and also the competitor, was also funded by NIH breast cancer SPORE grant at Lombardi Cancer Research Center to study the 80 kDa proteinase in human breast cancer progression. Because PI's Career Development Award only provided a limited funding (only part of PI's salary and no research funding provided), it is not easy for PI to compete with Dr. Dickson's lab on the proposed studies on 80 kDa proteinase. In the recent J. Biol. Chem. paper, Dr. Dickson's lab has developed a specific monoclonal antibody against the 80 kDa proteinase and partially characterized the enzyme (3).

Within the **similar** research area of breast cancer metastasis, the **new** revised research proposal will focus on the characterization of a novel breast cancer specific gene **BCSG1** in human breast cancer progression. Breast cancer evolves by clonal selection of cells that acquire multiple genetic changes. One proposed model for such accumulation suggests that breast cancer, like colon cancer (4), develops through defined morphologically distinguishable stages beginning with benign hyperplasia, progresses to atypical hyperplasia, which leads to a carcinoma *in situ*, and finally to an invasive carcinoma (5). Identification of quantitative changes in gene expression that occur in the breast cancer malignant progression, if sufficiently characterized, may yield novel molecular markers which may be useful in the diagnosis and treatment of human breast cancer. Within this context, we undertook a search, using the differential cDNA sequencing approach (6), for isolation of differentially expressed genes in the cDNA libraries from normal breast and breast

carcinoma. Of many putative differentially expressed genes, a breast cancer specific gene, BCSG1, which was expressed in high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker (6).

II. SPECIFIC AIMS AND HYPOTHESES

Introduction:

We have cloned a new breast cancer specific gene BCSG1 by differential cDNA sequencing. BCSG1, which was expressed in high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker. In preliminary studies, we found that: 1) BCSG1 expression is stage-specific in human breast epithelial cells as follows: BCSG1 is undetectable in normal or benign breast lesions, shows partial expression in ductal carcinoma *in situ*, but is expressed at an extremely high level in advanced infiltrating breast cancer; 2) overexpression of BCSG1 in MCF-7 human breast cancer cells stimulates cell growth in vitro and tumor growth in nude mice; 3) BCSG1 expression is dramatically suppressed by tumor growth inhibitor oncostatin M (OM), a cytokine predominantly produced by activated T cells and macrophages. Based on these findings, we propose the following hypotheses.

Hypotheses:

1. Overexpression of BCSG1 may correlate with clinical aggressiveness and indicate breast cancer malignant progression. The use of BCSG1 gene could be of importance in differentiating atypical proliferative breast lesions or noninvasive carcinoma *in situ* from malignant and invasive cancer and may be useful in screening of breast biopsies for potential abnormalities. In addition, if overexpression provides a therapeutic target, then BCSG1 may be useful in clinical management and treatment of breast cancer.
2. Alternations of BCSG1 expression may lead to an abnormal growth and malignant progression. Up-regulation of BCSG1 expression may facilitate breast cancer malignant progression from a benign breast or low a grade *in situ* carcinoma to the highly infiltrating carcinoma.
3. The expression of BCSG1 is regulated during breast cancer progression: up-regulated in the growth stimulatory environment and down-regulated in the growth inhibitory environment, such as by macrophage-derived tumor suppressing factor OM.

Specific aims

To test these hypotheses, we propose studies with the following Specific Aims:

SA1. Association of BCSG1 expression with breast cancer malignant progression. We will confirm and extend preliminary studies which suggest that expression of BCSG1 may indicate breast cancer progression. In this regard, we propose to have an assessment of the pathological importance of BCSG1 expression in the clinical realm relevant for human breast cancer development and progression. Specifically, we will determine if: a) BCSG1 expression is significantly different in less malignant non-Comedo type DCIS and more malignant Comedo type DCIS; b) BCSG1 expression is significantly different in benign hyperplasia and atypical hyperplasia.

SA2. Regulation of tumor growth and invasion and metastasis by BCSG1. We will confirm and extend preliminary studies which suggest that overexpression of BCSG1 stimulates breast cancer cell growth and invasion potentials. We will determine if BCSG1 overexpression (cDNA transfection) will convey an increased growth rate and invasion potential *in vitro* and tumorigenic and metastatic phenotypes in orthotopic nude mice model.

SA3. Molecular interactions between BCSG1 and other factors involved breast cancer growth and progression. We will begin to determine the mechanisms whereby BCSG1 expression is involved in breast cancer progression. We will determine the effects of broad range of growth factors and inhibitors on BCSG1 expression.

III. NEW STATEMENT OF WORK

SA1: Association of BCSG1 expression with breast cancer malignant progression
Year 2-4

Screening of BCSG1 expression in clinical breast specimens
- **a**. *in situ* hybridization analysis
- **b**. immunohistochemical analysis

SA2: Regulation of tumor growth and invasion and metastasis by BCSG1.
Year 2-4
Effects of BCSG1 overexpression on tumor growth and metastasis
- **a.** establishment of BCSG1 overexpressing breast cancer cell lines
- **b**. *in vitro* analysis of growth rate and invasive phenotypes of BCSG1 transfected clones
- **c.** analysis of tumorigenic and metastatic phenotypes in an *in vivo* orthotopic xenograft model

SA3: Regulation of BCSG1 expression.
Years 3-4
- **a.** Hormonal regulation
- **b.** Regulation by growth factors

IV. BACKGROUND AND SIGNIFICANCE

IV-1. Identification of genes differentially expressed in breast cancer versus normal breast.

There are two classes of genes affecting tumor development. Genes influencing the cancer phenotype that act directly as a result of changes (eg., mutation) at the DNA level, such as BRCA1, BRCA2, and p53, are called Class I genes. The Class II genes affect the phenotype by modulation at the expression level. Development of breast cancer and subsequent malignant progression is associated with alterations of a variety of genes of both classes. Many new predictive and prognostic factors have been proposed and studied for breast cancer. HER 2/neu positive tumors respond poorly to endocrine treatment (7-8). p53 alteration has an indication of poorer prognosis and poor response to tamoxifen (9-10). The lack of Nm23 expression has an indicative value of metastatic potential and poor prognosis in invasive ductal carcinoma (11). Cathepsin D, a protease suggested having a role in breast cancer, appears to affect the potential for invasive growth (12-13). Positive immunostaining of tumor sections with Factor VIII antibodies seems to be a marker for angiogenesis (14-16). It has been postulated that these tumors are targets for anti-angiogenesis drug treatment. Expression of the *mdr-1* gene is proposed to be an indicator of multidrug resistance (16). Poor response to endocrine therapy has been indicated for uPA/PAI-1, a plasminogen activator/inhibitor (17). Also receiving major attention is the familial breast cancer related genes, BRCA1 and BRCA2 (18-20).

Studies linked to the discovery of new genetic markers will provide new information leading to understanding of breast cancer development and progression. Identification of quantitative changes in gene expression that occur in the breast cancer malignant progression, if sufficiently characterized, may yield novel molecular markers which may be useful in the diagnosis and treatment of human breast cancer. We have described here a novel putative breast cancer specific gene BCSG1 that is overexpressed in advanced infiltrating breast cancer cells, but not in normal or benign breast lesion. The expression pattern of BCSG1 may be a meaningful marker in the development of breast cancer and subsequent malignant progression. In the preliminary studies, we demonstrated a stage-specific BCSG1 expression and an association of BCSG1 overexpression with clinical aggressiveness of breast cancers. The notion that the BCSG1 overexpression may lead to breast cancer malignant progression warrants further investigation.

IV-2. Alzheimer's disease (AD) amyloid protein.

It is interesting to note that the predicted amino acid sequences of BCSG1 gene shares high sequence homology with non-Aß component of Alzheimer's disease (AD) amyloid precursor protein (21). A neuropathological hallmark of AD is a widespread amyloid deposition resulting from beta-amyloid precursor proteins (beta APPs). Beta AAPs are large membrane-spanning proteins that either give rise to the beta A4 peptide (Aß fragment) (22) or a non-Aß component of AD amyloid (21) that is either deposited in AD amyloid plaques or yielding soluble forms. While the insoluble membrane-bound AD amyloid destabilizes calcium homeostasis and thus renders cells vulnerable to excitotoxic conditions of calcium influx resulting from energy deprivation or overexcitation (23), the soluble AD amyloid proteins are neuroprotective against glucose deprivation and glutamate toxicity, perhaps through their ability to lower the intraneuronal calcium concentration (24).

We currently do not know the significance of sequence homology between BCSG1 and AD amyloid protein. However, the recent studies indicate that amyloid protein is a potent stimulator for matrix metalloproteinase (MMP) (25). In these studies, the activities of MMP-2 and MMP-9 were increased significantly in neuronal cultures treated with amyloid protein. It is well established that the overproduction and unrestrained activity of MMPs and particular MMP-2 and MMP-9 has been linked to malignant conversion of a variety of different tumor cells (26-35) including mammary tumors (31-35). It is interesting to test whether BCSG1, an **amyloid-like** protein, stimulates MMP-2 and MMP-9 expression in breast cancer cells and leads to the more invasive phenotype. An elucidation of the reasons for BCSG1 overexpression in infiltrating breast cancer cells may shed some light on the pathogenesis of breast cancer progression.

IV-3. Suppression of breast cancer growth by oncostatin M (OM)

OM is a 28-kDa glycoprotein that originally was defined as a novel growth regulator based on its ability to inhibit growth of human melanoma cells (36). Researches conducted to date show that OM is predominantly produced by activated monocytes, macrophages, and T cells (36). Although the plasma concentration of OM in normal donors was below the EIA detection level (37), OM was detected in breast cyst fluid (38). It has been demonstrated that OM inhibits the cell growth of a variety of human breast cancer cells (39-40). The biological activities of OM in breast cancer cells are mediated by the OM-specific receptor, which is composed of gp130 as a binding subunit (40-41).

Growth inhibitory activity of OM is partially due to its functional antagonism of breast cancer mitogens including EGF, EGF-like growth factor, and bFGF (39). The proliferative activities of these growth factors were totally abolished by co-treatment of breast cancer cells with OM (39).

Examination of oncogene expression demonstrated that OM down regulates the c-myc gene. The c-myc protooncogene plays an important role in cell proliferation. It is possible that suppression of c-myc expression by OM results in a growth suppression of breast cancer cells. Since OM is produced by activated T cells and macrophages, a possible model of OM mediated growth suppression of tumors *in vivo* would involve infiltration of a large number of activated T cells and macrophages, which would then release OM *in situ* as a paracrine factor, thereby suppressing mitogen induced proliferation. However, in cells expressing little or no OM-specific receptor, *in situ* production of OM would have little or no growth inhibitory effect, thus allowing tumor progression.

Since the expression of BCSG1 may be involved in breast cancer progression, we are interested to see whether OM can alter the BCSG1 expression in breast cancer cells as one of the potential mechanisms underlying OM-mediated growth inhibition. It is interesting that OM strongly inhibits the BCSG1 gene expression, and this OM-induced suppression of BCSG1 is associated with OM-mediated growth inhibition.

V. WORK ACCOMPLISHED

V-1. Identification, cloning, and expression of BCSG1 (Please see attached reprint 1 for detailed description)

Summary. A direct differential cDNA sequencing approach was employed to identify genes differentially expressed in normal breast as compared with breast cancer. Of many putative differentially expressed genes, a breast cancer specific gene BCSG1, which was expressed in high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker. *In situ* hybridization analysis demonstrated a stage-specific BCSG1 expression as follows: BCSG1 was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but was expressed at an extremely high level in advanced infiltrating breast cancer. The predicted amino acid sequence of BCSG1 gene has a significant sequence homology to non-Aß fragment of Alzheimer's disease amyloid protein. BCSG1 overexpression may indicate breast cancer malignant progression from benign breast or *in situ* carcinoma to the highly infiltrating carcinoma.

V-2. Transcriptional suppression of BCSG1 by oncostatin M (OM) (Please see attached manuscript 2 for detailed description).

Summary. OM is an anti-tumor cytokine produced by activated T cells and macrophage (36). Growth inhibitory effect of OM on breast cancer cells has been demonstrated (39-40). Breast cancer cell lines that fail to respond to OM do not possess high-affinity OM bind sites (40), suggesting that the loss of OM receptor in breast cancer cells may contribute to escaping the OM-mediated growth inhibition. Since OM has an inhibitory and differentiative effect on H3922 cell growth, we examined the effect of OM on BCSG1 gene expression. A kinetic study showed that treatment of H3922 cells with OM initiated an immediate decrease of BCSG1 mRNA as early as 30 min. By 4 h, the level of BCSG1 mRNA was decreased to 70% of control, and by 24 h, the mRNA was completely undetectable. The effect of OM on BCSG1 was concentration-dependent with maximal suppression at concentration of 1 ng/ml. This OM-induced transcriptional suppression of BCSG1 gene is associated with OM-mediated growth inhibition. Results from nuclear run-on analysis and mRNA stability studies indicated that the observed OM-induced down-regulation of BCSG1 mRNA occurs at the transcriptional level. OM treatment of H3922 cells for 16 hours reduced the abundance of

actively transcribed BCSG1 mRNAs to 28.5% of that observed in control cells. These data were further supported by the finding that OM did not affect the stability of BCSG1 mRNA in actinomycin D-treated H3922 cells. The effect of OM on BCSG1 gene expression was not shared by other OM related cytokines including IL-6, IL-11, and LIF (leukemia inhibitory factor). These cytokines inhibited neither H3922 cell growth **nor** BCSG1 transcription. The dramatic suppression of BCSG1 expression by OM favors the potential role of BCSG1 as a mediator during breast cancer progression and may thus be involved in OM-induced growth inhibition.

V-3. Transfection of BCSG1 into human breast cancer cells.

Transfection and establishment of BCSG1 stable transfectants. In order to determine the effects of BCSG1 on primary tumor growth and invasion/metastasis, we have selected MCF-7 and MDA-MB-435 human breast cancer cell lines as recipients for BCSG1 mediated gene transfection because of: 1) their lack of detectable or BCSG1 transcript (Fig. 1 in reprint 1); 2) less aggressive behavior of MCF-7 cells can be used for studying potential stimulatory effect of BCSG1 on tumorigenesis; and 3) highly tumorigenic and aggressive MDA-MB-435 cells can be used as model for studying the potential effects of BCSG1 on invasion and metastasis. In order to facilitate the detection of micro-metastasis, MCF-7 cells have been previously transfected with Lac-Z (hygromycin B resistant) by PI's former colleague Dr. Mcleskey at Lombardi Cancer Research Center (42). Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo clones), or the same vector containing a full-length BCSG1 cDNA (BCSG1 clones). Clones were initially screened by *in situ* hybridization on slides with a specific BCSG1 antisense probe (data not shown), and the positive clones were subjected to Northern blot analysis. MDA-MB-435 clones expressing BCSG1 were named as **BC-435** clones, and the control neo transfected cells were named as **neo-435** clones; MCF-7 clones expressing BCSG1 were named as **BC-MCF** clones, and neo transfected cells were named as **neo-MCF** clones.

Fig. 1 shows the Northern blot analysis of BCSG1 expression in selected clones. All selected BC-435 clones (Fig. 1A) and BC-MCF clones (Fig. 1B) expressed BCSG1 mRNA transcripts. In contrast, none of the neo-435 and neo-MCF clones produced any detectable BCSG1 transcripts. No changes in morphology were observed in these clones.

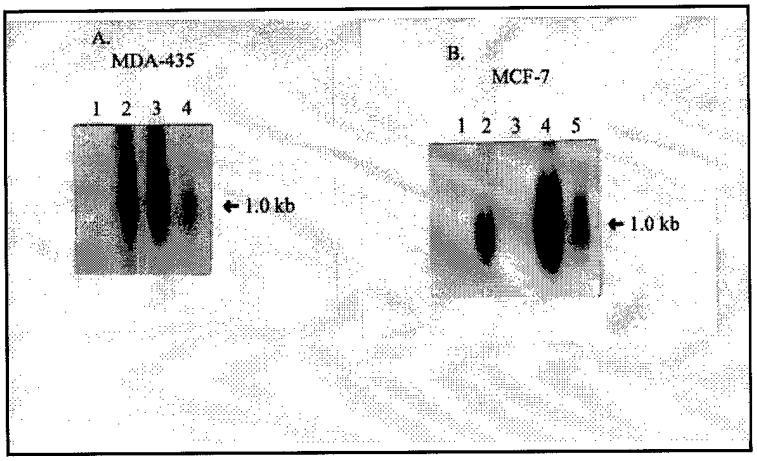

Fig. 1. Northern analysis of BCSG1 expression. A. MDA-MB-435 cells. 1: neo-435-1; 2: BC-435-3; 3: BC-435-2; 4: BC-435-1. B. MCF-7 cells. 1: neo-MCF-1; 2: BC-MCF-2; 3: neo-MCF-2; 4: BC-MCF-6; 5: BC-MCF-4.

In vitro growth of BCSG1 transfected cells. To determine whether BCSG1 overexpression affects the growth of the transfected cells, the growth rates of BCSG1 positive MCF-7 clones (BC-MCF-2 and BC-MCF-6) were compared to that of BCSG1 negative MCF-7 cells (neo-MCF-1 and neo-MCF-2) in a monolayer culture. Cell growth was stimulated approximately 3.2-fold ($p < 0.001$ by Student's *t-test*) in the BC-MCF-2 and BC-MCF-6 cells compared to neo-MCF-1 and neo-MCF-2 cells (Fig. 2). We also compared the growth rates of BCSG1 positive MDA-MB-435 clones (BC-435-2 and BC-435-3) vs. BCSG1 negative MDA-MB-435 clones (neo-435-1 and neo-435-2). No significant differences in growth rate were observed among BCSG1 positive and BCSG1 negative clones (data not shown). The lack of the stimulatory effect of BCSG1 on more malignant MDA-MB-435 cells suggests that these cells are less sensitive to the stimulatory factors such as BCSG1, which may be due to their hormone-independent and overloaded growth advantages. These results suggest that up-regulation of BCSG1 expression enhances the growth of less malignant breast cancer cells such as hormone-dependent MCF-7 cells.

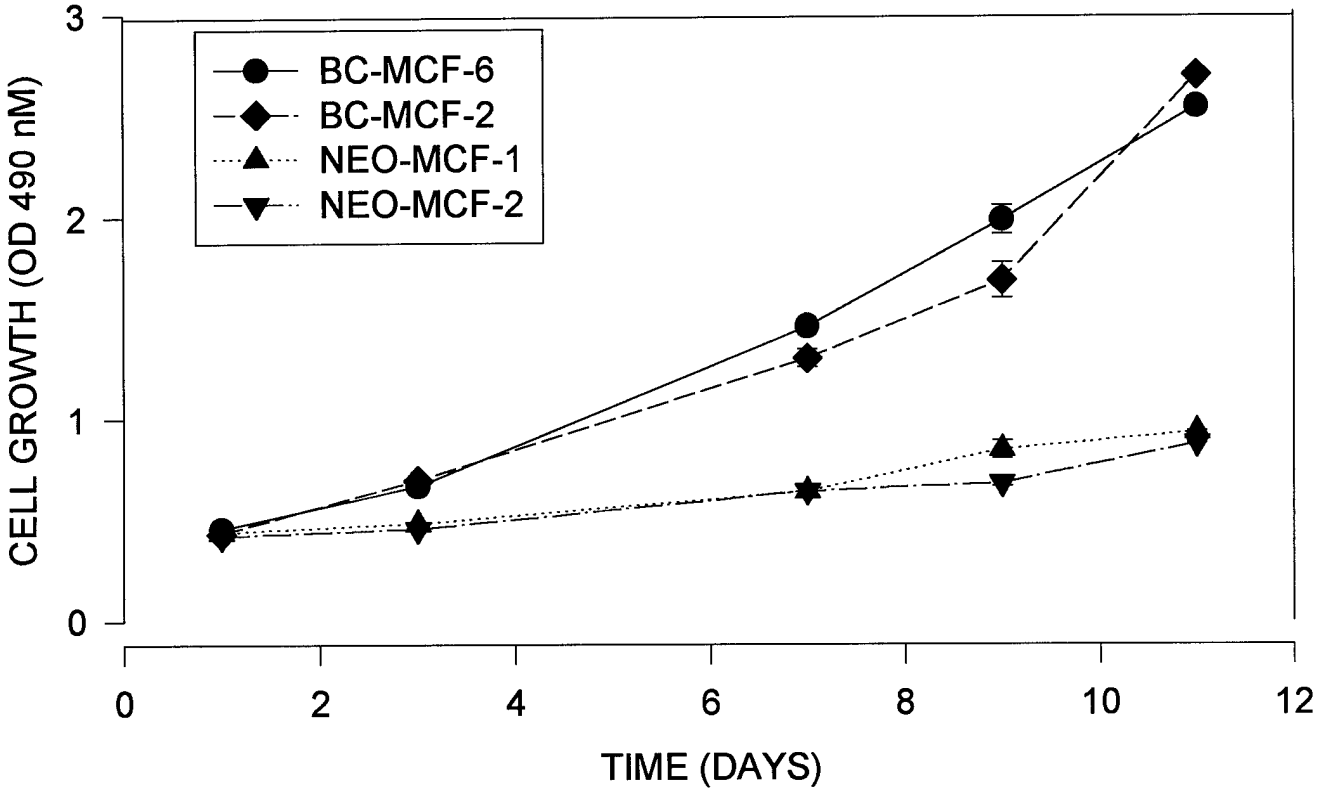

Fig. 2. Effect of BCSG1 overexpression on growth. Exponentially growing cultures of different MCF-7 clones were detached with trypsin, and the trypsin was neutralized with DMEM-10% serum. Cells were counted, diluted, and seeded in triplicate at 3,000 cells per well (24-well plate) in 1 ml DMEM-5% serum. The growth rates of BCSG1 positive clones were significantly greater than that of BCSG1 negative clones, with the most fast growing rate for BC-MCF-6 clones. At day 11, both BC-MCF-2 and BC-MCF-6 cells reached confluence. Cell growth was measured using CellTiter 96™ Aqueous Non-Radioactive cell proliferation Assay Kit (Promega). The number represents the mean ± SE of three cultures.

Stimulation of invasiveness of MDA-MB-435 cells by BCSG1. The higher level expression of BCSG1 in advanced infiltrating breast carcinomas relative to the non-invasive *in situ* carcinomas (Fig. 4 in reprint 1) suggests that up-regulation of BCSG1 during the malignant progression may facilitate the cellular invasion and metastasis. In fact, the amyloid protein, which BCSG1 has great sequence homology with, has been recently demonstrated to be a strong stimulator for MMP-2 and MMP-9 expression in astrocytes (25). We carried out a pilot *in vitro* study to investigate if up-regulation of BCSG1 would increase the invasiveness of MDA-MB-435 cells.

We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of BCSG1 on cell invasion. We picked two BCSG1 negative lines (neo-435-1 and neo-435-2) and two BCSG1 positive lines (BC-435-2 and BC-435-3). Both neo-435-1 and neo-435-2 cells were moderately invasive. At the end of 20-h incubation, about 1.5% of neo-435-1 cells and 1.2% of neo-435-2 cells had crossed the Matrigel barrier. A significant stimulation in invasiveness was noted in two BCSG1 expressing clones with percentages of invasion for BC-435-2 and BC-435-3 being 4.1% and 2.9%, respectively (Fig. 3).

MCF-7 cells are not invasive at all in the Matrigel invasion assay. Although we haven't tested BCSG1 transfected MCF-7 cells, we do not expect the stimulatory effect of BCSG1 on the invasiveness of MCF-7 cells, simply because that these cells tend to grow as clumps but not individual cells. A cluster of cells can't move through the Matrigel barrier.

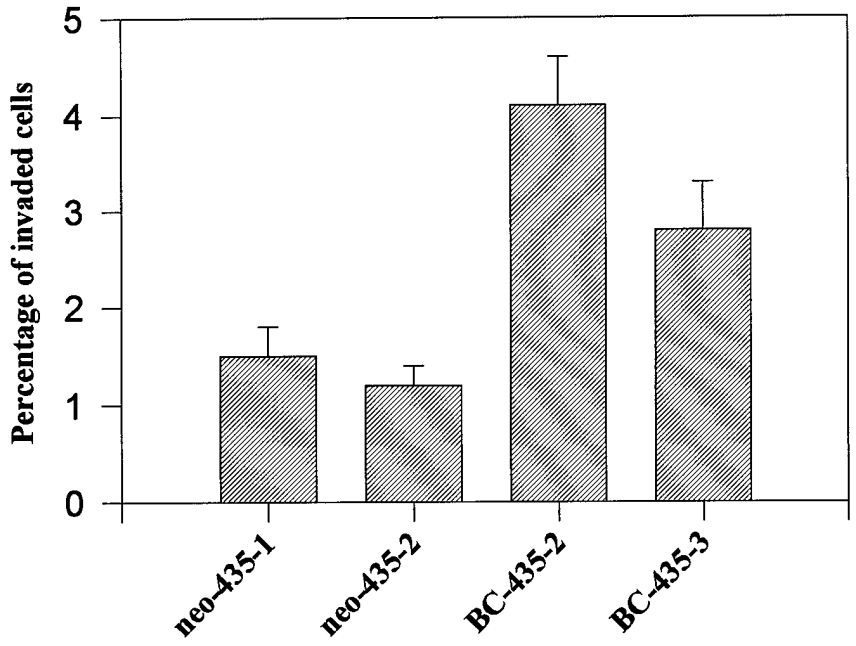

Fig. 3. Stimulation of invasiveness of MDA-MB-435 cells by BCSG1. Ten-μm polycarbonate membranes were coated with 4 mg/ml growth factor-reduced Matrigel in medium as we described previously (43,45). The cells were seeded at a density of 50,000 cells/ml/well in DMEM containing 5% serum. After incubation in a humidified incubator with 5% CO_2 at 37^{0}C for 24 h, the medium as well as the cells were removed from the bottom chambers and counted using a Zeiss microscope. All values were expressed as a percentage of invaded cells. The numbers represent the means $\pm$ SEs of three cultures.

Tumorigenesis in orthotopic nude mice model. Since the BCSG1 expressed MCF-7 clones grow faster than that of BCSG1 negative clones, we are interested in studying the effect of BCSG1 expression on tumorigenicity in an orthotopic nude mouse model. For a pilot experiment, we have picked two BCSG1 positive clones: BC-MCF-2, BC-MCF-6; and two BCSG1 negative clones: neo-MCF-1 and neo-MCF-2. Since the tumorigenesis of MCF-7 cells are hormone-dependent and less aggressive, and we don't know if the overexpression of BCSG1 changes their hormonal response status, we used the intact female nude mice (not ovariectomized mice). In addition, as we previously reported (43), in order to increase the tumor incidence of hormone-dependent cells, the Matrigel was used to facilitate the tumor take.

After a lag phase of approximately 7 days, 12 out of 14 injections of BCSG1 **positive** clones formed tumors. However, 8 out of 14 injections of BCSG1 **negative** clones formed small non-progressive tumors that subsequently regressed after 15 days, and only 6 out of 14 injections developed solid tumors at the time of scarification. Like what we demonstrated in the *in vitro* growth, the tumor growth of MCF-7 cells was also stimulated in the BCSG1 transfected clones. The magnitude of stimulation of tumor size is correlated with the levels of BCSG1 expression in the BC-MCF clones. The size of the **highest** BCSG1 expressing BC-MCF-6 tumors was **2.5-fold** over that of neo-MCF-1 tumors and **4-fold** over that of neo-MCF-2 tumors. The tumor growth of the lower BCSG1 expressing BC-MCF-2 cells was also significantly increased, with a **1.7-fold** and **2.7-fold** increase over that of neo-MCF-1 and neo-MCF-2 tumors, respectively.

Table 1. Effects of BCSG1 expression on tumor incidence and tumor size of MCF-7 cells

Group	*MCF-7 clones*	*Tumor Vol (mm^3) of Primary Size*	*Tumor Incidence Tumor/Total (%)*
1	neo-MCF-1	89 ± 38	3/7 (43%)
	BC-MCF-6	227 ± 51	7/7 (100%)
2	neo-MCF-2	56 ± 22	3/7 (43%)
	BC-MCF-2	154 ± 41	5/7 (71%)

Nude mouse tumorigenic assay was performed as we previously described (43-44). Briefly, the MCF-7 clones were grown to 80-90% of confluence in 150 cm^2 dishes and were harvested by incubation with 5 mM EDTA in PBS. The EDTA was neutralized with medium containing serum. The cells were washed twice with serum-free medium, counted, and re-suspended in the cold serum-free DMEM (4°C) at a concentration of 8 x 10^7 cells/ml. The cell suspension was then equally mixed with pre-cold Matrigel (Collaborative) to give a final concentration of 4 x 10^7 cells/ml. Approximately 5 x 10^6 cells (0.1 ml) were injected at day 1 into the 5-6 week old female athymic nude mice (Frederick Cancer Research and Development Center, Frederick, MD). Each animal received two injections, with BC-MCF cells on the left and neo-MCF cells on the right, in the mammary fat pads between the first and second nipples. The animals were ear tagged. Primary tumor growth was assessed by

measuring the volume of each tumor at weekly intervals. Tumor size was determined at intervals by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point. Animals were sacrificed between at 35 days after injection. Statistical comparison for pooled BCSG1 positive clones relative to pooled BCSG1 negative clones indicates $P < 0.01$ for the mean tumor size.

The above preliminary data suggest a role of BCSG1 as positive regulator for both tumor growth and invasiveness. Up-regulation of BCSG1 may facilitate the breast cancer progression. However, we realize that the different growth rates and invasion potentials between two BCSG1 positive and two BCSG1 negative clones may be due to the endogenous differences of individual clones but not BCSG1. The experiments need to be repeated for more clones.

V. CONCLUSION

Studies linked to the discovery of new genetic markers will provide new information leading to understanding of breast cancer development and progression. We have described here a novel putative breast cancer specific gene BCSG1 that is overexpressed in advanced infiltrating breast cancer cells, but not in normal or benign breast lesions. In the preliminary studies, we demonstrated a stage-specific BCSG1 expression and an association of BCSG1 overexpression with clinical aggressiveness of breast cancers. Overexpression of BCSG1 human breast cancer cells stimulates cell growth in vitro and tumor growth in nude mice. BCSG1 expression is dramatically suppressed by tumor growth inhibitor oncostatin M (OM), a cytokine predominantly produced by activated T cells and macrophages. The expression pattern of BCSG1 may be a meaningful marker in the development of breast cancer and subsequent malignant progression. The notion that the BCSG1 overexpression may lead to breast cancer malignant progression warrants further investigation. We will study the biological functions of BCSG1 on breast cancer growth and metastasis; to define its range of expression and begin to determine the mechanisms whereby BCSG1 expression is involved in breast cancer progression. The knowledge gained from the proposed studies allow us to target specific pathways contributory to the breast cancer progression.

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Identification of a Breast Cancer-specific Gene, *BCSG1*, by Direct Differential cDNA Sequencing[1]

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ABSTRACT

A high-throughput direct-differential cDNA sequencing approach was employed to identify genes differentially expressed in normal breast as compared with breast cancer. Approximately 6000 expressed sequence tags (ESTs) from cDNA libraries of normal breast and breast carcinoma were selected randomly and subjected to EST-sequencing analysis. The relative expression levels of more than 2000 unique EST groups were quantitatively compared in normal *versus* cancerous breast. Of many putative differentially expressed genes, a breast cancer-specific gene, *BCSG1*, which was expressed in high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker. *In situ* hybridization analysis demonstrated stage-specific BCSG1 expression as follows: BCSG1 was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but was expressed at an extremely high level in advanced infiltrating breast cancer. The predicted amino acid sequence of *BCSG1* gene has a significant sequence homology to the non-amyloid β protein fragment of the Alzheimer's disease amyloid protein. BCSG1 overexpression may indicate breast cancer malignant progression from benign breast or *in situ* carcinoma to the highly infiltrating carcinoma.

INTRODUCTION

The onset and progression of breast cancer is accompanied by multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (1). Our hypothesis is that many of these quantitative genetic changes manifest themselves as alterations in the cellular complement of novel transcribed mRNAs. Identification of these mRNAs, if sufficiently characterized, could provide clinically useful information for patient management and prognosis while enhancing our understanding of breast cancer pathogenesis. Although pathological end points such as tumor size, lymph node status, and status of estrogen receptor and progesterone receptor remain the most useful guides in prognosis and in selecting treatment strategies for breast cancer (2), there is a need to further investigate the molecular mechanisms that determine the properties of an individual tumor, *e.g.*, probability of metastasis. Although numerous prognostic factors have now been identified, few have contributed to defining the clinical response to therapy.

Identification of quantitative changes in gene expression that occur in the malignant mammary gland, if sufficiently characterized, may yield novel molecular markers that may be useful in the diagnosis and treatment of human breast cancer. Several differential cloning methods, such as differential display PCR and subtractive hybridization, have been used to identify the genes differentially expressed in breast cancer biopsies, as compared to normal breast tissue controls (3–7). However, these investigations have involved the relatively time- and labor-intensive steps of subcloning, library screening, and cDNA sequencing of individual genes (4, 8). On the other hand, creation of libraries is a rapid method used to identify or "tag" sequences that are expressed in specific tissues (9, 10). Since the introduction of the EST[3] sequencing approach, many novel human genes have been discovered (9, 10). The advantage of this methodology, compared to isolation and sequencing of individual cDNAs, is that a large number of sequences can be "catalogued" with small amounts of sequencing data.

With the availability of tens of thousands of ESTs, researchers now shift their attention to the unveiling of the expression profile of individual genes or patterns of genes in normal *versus* diseased states. Several newly developed strategies, such as the serial analysis of gene expression (11) and cDNA microarray (12) methods, have demonstrated potential for broad application for quantitative analysis of differential patterns of gene expression. Within this context, we undertook a search, using the differential cDNA sequencing approach, for isolation of differentially expressed ESTs and the possible presence of the new marker genes for breast cancer. In this initial report, we describe a novel BCSG named *BCSG1* that is overexpressed in advanced infiltrating breast cancer cells but not in normal or benign breast lesion. The expression pattern of BCSG1 may be a meaningful marker in the development of breast cancer.

MATERIALS AND METHODS

Reagents. Restriction enzymes, T7 polymerase, random primer DNA labeling kit, and digoxigenin-labeled nucleotides were obtained from Boehringer Mannheim (Indianapolis, IN). [^{32}P]dATP was purchased from Amersham Corp.

Differential cDNA Sequencing. We have used EST analysis to search for new genes differentially expressed in breast cancer *versus* normal breast tissue. A data base containing approximately 500,000 human partial cDNA sequences (ESTs) has been established in a collaborative effort between the Institute for Genomic Research and Human Genome Sciences, Inc., using high-throughput automated DNA sequence analysis of randomly selected human cDNA clones (10). RNAs from a stage III breast carcinoma and patient-matched normal breast were isolated and subjected to preparation of cDNA libraries. EST-automated DNA sequence analysis was performed on randomly selected cDNA clones. Both libraries had about 60% novel gene sequences, which did not match exactly to published human genes. A total of 3048 ESTs from breast cancer cDNA library and 2886 ESTs from the normal breast cDNA library were randomly picked and sequence analyzed. The ESTs with overlapping sequences were grouped into unique EST groups, with each EST group representing a gene or a family of sequence-related genes. Each unique EST group without overlapping sequences was analyzed for its relative expression by examining the number of expressed individual ESTs in the libraries of normal *versus* diseased tissues. There were more than 2200 EST groups that were analyzed for quantitative comparison of EST "hits" in the pair of cDNA

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[3] The abbreviations used are: EST, expressed sequence tag; BCSG, breast cancer-specific gene; Aβ, amyloid β protein; AD, Alzheimer's disease; DCIS, ductal carcinoma *in situ*.

Table 1 *Partial list of differentially expressed genes in normal versus cancerous breasts identified by differential cDNA sequencing*

Complementary DNA libraries were established from a stage III breast carcinoma and patient-matched normal breast. A total of 5934 ESTs were randomly picked and sequence analyzed. More than 2200 distinctive EST groups were analyzed for quantitative comparison of EST hits in the pair of cDNA libraries from breast cancer *versus* normal breast as described in "Materials and Methods." The same EST groups were also analyzed by examining the tissue-specific expression against the total of 500,000 ESTs from a variety of different cDNA libraries. Only a unique EST group with more than three breast-specific EST hits was listed, and the rest of the several dozen EST groups with fewer than four breast-specific EST hits were omitted in this list.

Genes more abundant in breast cancer

	ESTs	
Class I Genes	Cancer	Normal
Breast basic conserved gene	33	9
Cathepsin D	5	1
M_r 67,000 laminin receptor	4	0
Elongation factor 1	13	5

Genes more abundant in normal breast

	ESTs	
Class II Genes	Cancer	Normal
Matrix Gla protein	0	8
M_r 23,000 highly basic protein	3	11

Genes as breast-specific and differentially expressed

	ESTs		
Class III Genes	NB[a]	BC[b]	All tissues
BCSG1	1	6	8[c]
BCSG2	0	7	7
BCSG3	0	5	5
BCSG4	4	0	4
BCSG5	0	4	4

[a] normal breast; [b] breast cancer; [c] seven ESTs from breast libraries and one EST from brain library.

libraries from normal breast *versus* breast cancer by examining the expression of individual EST sequences. The number of EST hits in the libraries reflects the relative expression or mRNA transcript copy numbers of the EST. This direct differential cDNA sequence, utilizing the direct EST sequencing analysis simultaneously on a pair of cDNA libraries made from normal breast and breast cancer tissue, was used to study the expression profile of individual genes and patterns of genes in normal breast *versus* breast cancer tissue.

Tissue-specific Expression Analysis. Analysis of relative expression of breast-derived ESTs *versus* their expression in other tissues was performed. The differentially expressed EST groups identified by differential cDNA sequence were analyzed for tissue-specific expression against the total of 500,000 ESTs from a variety of different cDNA libraries.

Northern Analysis. Total RNA was extracted from tissues according to the method of Chomczynski and Sacchi (13). The RNA from human breast cancer cells was prepared using the RNA isolation kit RNAzol B (Tel-Test, Inc.) based on the manufacturer's instruction. Equal aliquots of RNA were electrophoresed in a 1.2% agarose gel containing formaldehyde and transferred to nylon membrane (Boehringer Mannheim). The membrane was prehybridized with ExpressHyb hybridization solution (Clontech, Inc.) at 68°C for 30 min. The hybridization was carried out in the same solution with ^{32}P-labeled BCSG1 probe (1.5×10^6 cpm/ml) for 1 h at 68°C. The membrane was then rinsed in 2× SSC containing 0.05% SDS three times for 30 min at room temperature, followed by two washes with 0.1× SSC containing 0.1% SDS for 40 min at 50°C. The full-length BCSG1 cDNA was isolated from the Bluescript vector following *Eco*RI and *Xho*I digestion and was used as a template for preparation of a random-labeled cDNA probe.

***In Situ* Hybridization.** *In situ* hybridization was carried out as described (14). Briefly, deparaffinized and acid-treated sections (5 μm thick) were treated with proteinase K, prehybridized, and hybridized overnight with digoxigenin-labeled antisense transcripts from a BCSG1 cDNA insert. The BCSG1 antisense probe is a 550-bp full-length fragment. The probe was generated by a *Pst*I cut of BCSG1 cDNA plasmid and followed by T7 polymerase. Hybridization was followed by RNase treatment and three stringent washings. Sections were incubated with mouse antidigoxigenin antibodies (Boehringer Mannheim) followed by the incubation with biotin-conjugated secondary rabbit antimouse antibodies (DAKO). The colorimetric detections were performed with a standard indirect streptavidin-biotin immunoreaction method using the Universal LSAB Kit (DAKO) according to the manufacturer's instructions.

RESULTS

Molecular Cloning of BCSG1 cDNA. We generated cDNA libraries from breast cancer biopsy specimens and patient-matched normal breast and analyzed these libraries by EST sequencing. Approximately 6000 ESTs were analyzed and assigned to different groups based on sequence overlapping, and 2200 unique EST groups were first analyzed for relative expression in the cDNA libraries from normal breast *versus* breast cancer tissue and then subjected to tissue-specific expression by examining the tissue origins of individual EST sequences against a large population of ESTs derived from a variety of different tissue types. As a result, we identified three classes of EST groups that were differentially expressed in normal breast *versus* breast cancer tissue. As a demonstration of this approach, Table 1 shows a partial list of three classes of genes that are differentially expressed in normal breast *versus* breast cancer tissue. Class I represents the genes more abundant in breast cancer than in normal breast and includes cathepsin D, a well-studied steroid regulated extracellular matrix-degrading proteinase (15–17). Cathepsin D is thought to play a role in breast cancer metastasis (15–17) and has been proposed as a prognostic marker in breast cancer progression (18–21). As listed, there were five cathepsin D ESTs sequenced in the breast cancer cDNA library and only one EST in the normal breast cDNA library. Another proposed breast cancer metastasis-related gene and a prognostic marker for breast cancer, M_r 67,000 laminin receptor (22–26), was also picked up in this class by the differential cDNA sequencing approach. Class II represents genes that are more abundant in normal breast than in breast cancer.

```
1    M D V F K K G F S I A K K G V V G A V E    BCSG1
1    M D V F M K G L S K A K E G V V A A A E    *Human AD Amyloid

21   K T K Q G V T E A A E K T K E G V M Y V    BCSG1
21   K T K Q G V A E A A G K T K E G V L Y V    *Human AD Amyloid

41   G A K T K E N V V Q S V T S V A E K T K    BCSG1
41   G S K T K E G V V H G V A T V A E K T K    *Human AD Amyloid

61   E Q A N A V S K A V V S S V N T V A T K    BCSG1
61   E Q V T N V G G A V V T G V T A V A Q K    *Human AD Amyloid

81   T V E E A E N I A V T S G V V R K E D L    BCSG1
81   T V E G A G S I A A A T G F V K K D Q L    *Human AD Amyloid

101  R P S A P Q Q E G E A S K E K E E V A E    BCSG1
101  G K N E E G A P Q E G I L E D M P V D P    *Human AD Amyloid

121  E A Q S G G D                             BCSG1
121  D N E A Y E M P S E E G Y Q D Y E P E A    *Human AD Amyloid
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*Non-Aß component of Alzheimer's disease (AD) Amyloid

Fig. 1. Comparison of the predicted amino acid sequence with the sequence of non-Aβ component of AD amyloid protein using SwissProt. After optimal alignment using the clustal method of the MegAlign Program from the DNASTAR software package, the putative protein shows a 54% sequence identity with the non-Aβ fragment of human AD amyloid protein. Conserved amino acids are underlined.

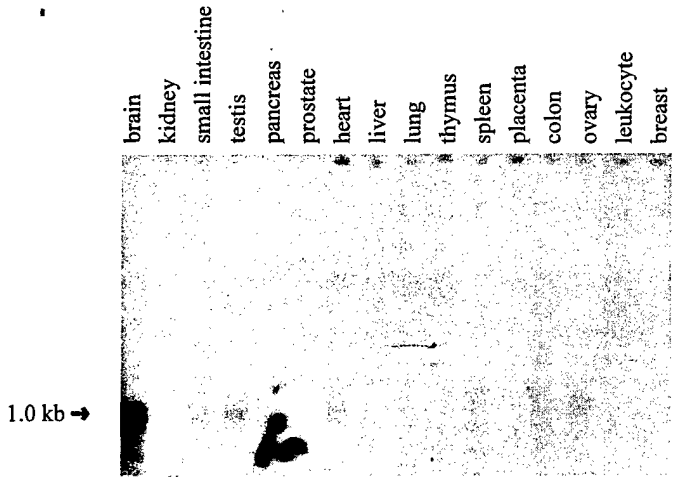

Fig. 2. The expression of *BCSG1* gene in a variety of normal human adult tissues. Twenty μg of total RNA from each of the above tissues were analyzed in Northern blot using a random primer probe. A strong hybridizing band of about 1 kb was recognized in the lane corresponding to RNA from adult brain. A weak 1-kb transcript was also detected in testis, heart, spleen, colon, and ovary.

Although the genes in classes I and II are differentially expressed in normal breast *versus* breast cancer tissue, these genes are not unique to breast tissues. Class III is a special group of genes that are selectively expressed in breast relative to other tissue types. The tissue-specific expression of the unique gene was searched against approximately 500,000 ESTs using the BLAST program (27). None of these BCSGs except the first one matched with any sequences in public gene sequence databases. The automated screening revealed a group of eight ESTs encoding a novel *BCSG1* gene from the partial cDNA database containing approximately 500,000 ESTs. Of the eight distinctive EST clones in BCSG1, seven of them were discovered in breast cDNA libraries and only one in a brain library. Of the seven EST clones discovered in the breast cDNA libraries, six of them were identified in the breast tumor library and only one in the normal breast library. BCSG1 was chosen for analysis as a first putative breast cancer marker gene because (*a*) its sequence has been matched with the sequence in the public gene sequence database; and (*b*) most of the individual EST sequences in BCSG1 were derived from a breast tumor cDNA library. After sequencing analysis of all six EST clones derived from the breast cancer library, one EST clone was found to have a complete full-length sequence. The open reading frame of the resulting full-length gene is predicted to encode a 127-amino acid polypeptide. Comparison of the predicted amino acid sequence with the sequence of a similar human protein is shown in Fig. 1. After optimal alignment, the putative BCSG1-encoded protein shows 54% sequence identity with the recently cloned non-Aβ fragment of human AD amyloid protein (28).

Tissue Expression. The expression of *BCSG1* gene in a variety of normal human tissues were analyzed by Northern blotting (Fig. 2). As expected, the Northern blot showed that BCSG1 was abundantly expressed as a 1-kb transcript in brain, which is the rich source for the *AD* amyloid family gene. Similar bands with much lower accumulations in their relative intensities were also obtained in ovary, testis, colon, and heart. By contrast, none of them was present in other specimens analyzed, such as breast, kidney, liver, prostate, lung, small intestine, thymus, and placenta.

[4] J. Liu, M. J. Spence, P. M. Wallace, K. Forcier, I. Hellstrom, and R. T. Vestal. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth, antagonism of growth factors, and down regulation of c-*myc* proto-oncogene, submitted for publication.

Expression of BCSG1 in Human Breast Cancer Cells. In an attempt to evaluate the potential biological significance of BCSG1 on human breast cancer development and progression, we studied *BCSG1* gene expression in human breast cancer cells. Northern blot (Fig. 3) detected the 1-kb BCSG1 transcript in two of four lines derived from pleural effusion and four of four lines detected from ductal infiltrating carcinomas. Among these lines, H3922 expressed the highest level of BCSG1 mRNA. The absence of BCSG1 mRNA in some breast cancer cell lines may suggest that the expression of *BCSG1* gene requires specific *in vivo* conditions, or that it is induced by interactions between the tumor cells and stromal cells.

To localize the cellular source of the BCSG1 expression and to further assess the biological relevance of the overexpression of BCSG1 in breast cancers, we next performed *in situ* hybridization on fixed breast sections from 20 infiltrating carcinomas, 15 DCISs, and 18 benign breast lesions, including 5 reduction mammoplasty specimens, 8 breast hyperplasias, and 5 fibroadenomas. In these experiments, we examined two aspects of BCSG1 expression, including the tissue localization (stromal *versus* epithelial) and the correlation of BCSG1 expression and breast cancer malignant phenotype. There was a wide variation in staining intensity for BCSG1 expression among the human breast cancer specimens. Because the colorimetric *in situ* hybridization is not quantitative, the tissue samples were classified into either positive or negative staining for BCSG1 expression; no attempt was made to differentiate the levels of expression of BCSG1 among positive-staining specimens. The negative cases were confirmed with at least two independent experiments. All stainings were reviewed by at least two people. Fig. 4 shows a representative *in situ* hybridization for BCSG1. We found a strongly positive BCSG1 hybridization in neoplastic epithelial cells of highly infiltrating breast carcinomas (Fig. 4, *A* and *B*). The expression of BCSG1 mRNA was detectable in the neoplastic epithelial cells in 17 of 20 infiltrating breast carcinomas. No expression of BCSG1 was detected in the stromal cells. In contrast, expression of BCSG1 was absent in 16 out of 18 cases of normal or benign breast lesions. A representative negative staining of BCSG1 in normal ductal breast epithelial cells (Fig. 4*E*), a benign proliferative breast lesion (Fig. 4*F*), and a benign fibroadenoma (Fig. 4*G*) are presented. Furthermore, as demonstrated in Fig. 4*B* for a highly invasive breast carcinoma, no detectable signal of BCSG1 expression was evident in the residual normal lobular breast epithelial cells, although the surrounding invasive breast carcinoma cells were stained positive for BCSG1 expression. The presence of BCSG1 transcript in human breast tissue and its overexpression in breast carcinomas are consistent with our differential cDNA sequencing cloning strategy, which suggests a possible

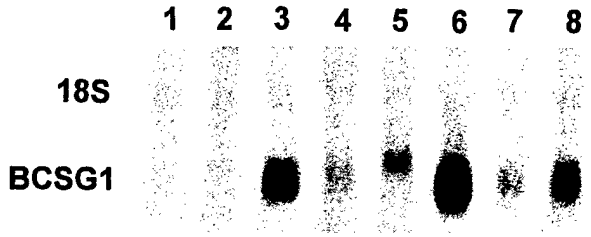

Fig. 3. Northern blot analysis of BCSG1 expression in human breast cancer cell lines. Total RNA was isolated and analyzed (20 μg/*Lane*) by Northern blot. After hybridization and washing, the filter was exposed to X-ray film for 48 h. The integrity and the loading control of the RNAs were ascertained by direct visualization of the 18 S rRNA in stained gel. *Lane 1*, H3396 (derived from pleural effusion); *Lane 2*, MCF7 (derived from pleural effusion); *Lane 3*, SKBR-3 (derived from pleural effusion); *Lane 4*, MDA-MB-231 (derived from pleural effusion); *Lane 5*, H3914 (derived from infiltrating ductal carcinoma); *Lane 6*, H3922 (derived from infiltrating ductal carcinoma); *Lane 7*, ZR-75–1 (derived from infiltrating ductal carcinoma); *Lane 8*, T47D (derived from infiltrating ductal carcinoma). Cell lines T47D, ZR-75–1, SKBR-3, MCF-7, and MDA-MB-231 are from American Type Culture Collection; all other lines were isolated initially at Bristol-Myers Squibb Pharmaceutical Research Institute.[4]

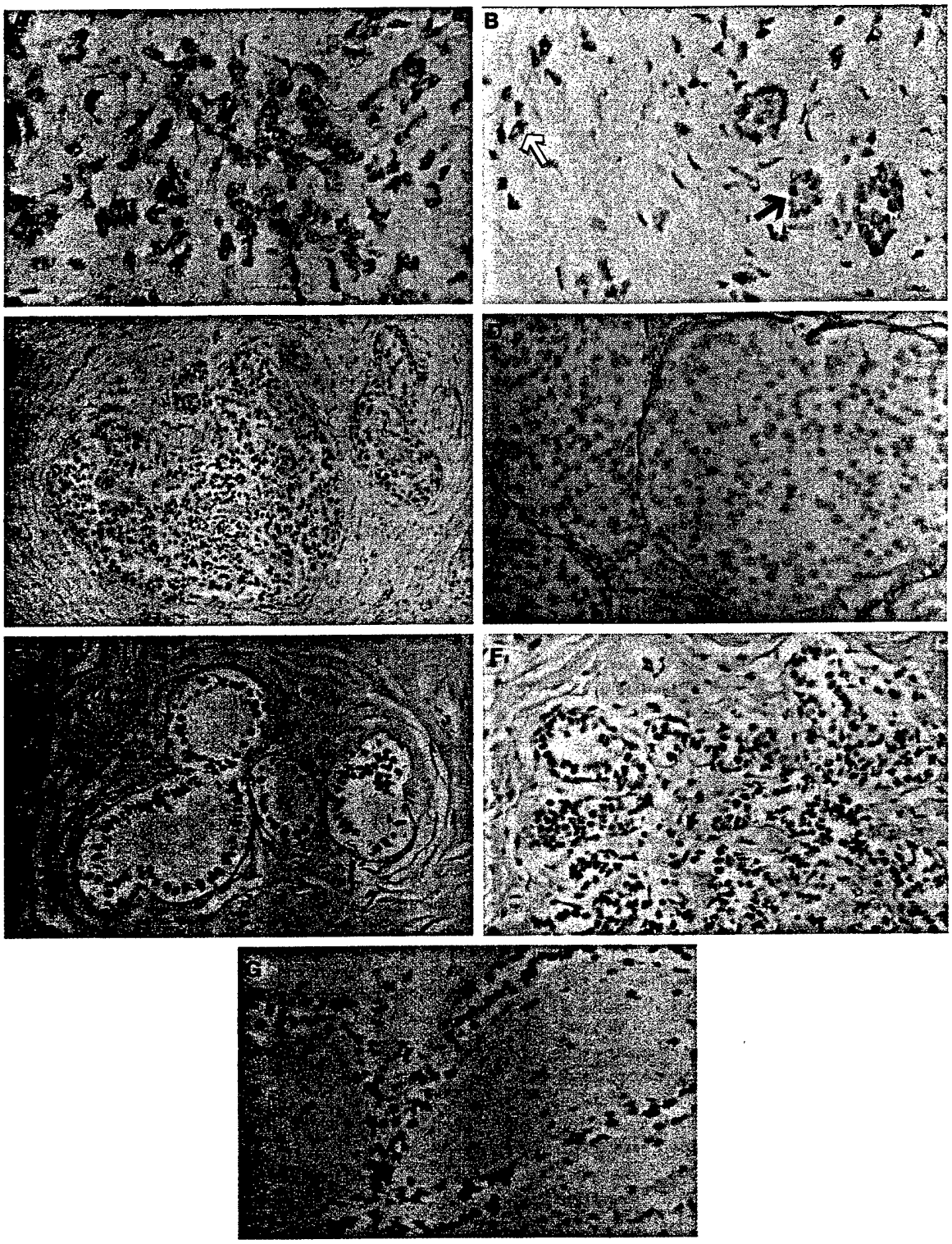

Fig. 4. *In situ* hybridization analysis of BCSG1 expression in human breast. Cells labeled with *brown* indicate *BCSG1* gene expression. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. *A*, a highly infiltrating breast carcinoma showed a very strong BCSG1 expression in virtually every malignant cell. *B*, high-magnification view of breast cancer cell invasion to normal lobule. *Solid arrow*, negatively stained residual normal lobular epithelial cells; *open arrow*, positively stained invasive cancer cells. *C*, Comedo-type DCIS showing BCSG1 staining. *D*, negative staining of BCSG1 in a non-Comedo-type DCIS. *E*, negative staining of normal ductal epithelial cells. *F*, negative staining of epithelial cells in a benign hyperplasia. *G*, negative staining of a benign fibroadenoma.

role or a biomarker of up-regulation of BCSG1 in the development of breast cancer.

It is interesting to note that although a strong BCSG1 signal was easily detected in the malignant breast epithelial cells of infiltrating breast carcinoma, the *in situ* carcinomas showed different BCSG1 expression patterns. Among 15 DCISs (8 are Comedo type and 7 are non-Comedo type), 8 specimens stained negatively (Fig. 4*D*) and 7 specimens were positive (Fig. 4*C*). Interestingly, six of seven BCSG1-positive DCIS samples were Comedo-type DCIS, and only one was non-Comedo type; among the BCSG1-negative specimens, there were six non-Comedo-type DCISs and only two Comedo-type DCISs. These results, which demonstrated a stage-specific BCSG1 expression

from virtually no detectable expression in normal or benign breast to partial expression (7 of 15) in the *in situ* breast carcinoma and to the high expression (17 of 20) in the infiltrating malignant breast carcinomas, suggest an association of BCSG1 expression with breast cancer malignant progression. On the basis of this BCSG1 expression pattern, we propose that BCSG1 may be used potentially as a breast cancer progression marker.

DISCUSSION

More than 190,000 new cases of breast cancer are diagnosed in the United States every year, with incidence increasing by approximately 1% annually (29, 30). Studies linked to the discovery of new genetic markers will provide new information leading to the understanding of breast cancer development and progression. There are two classes of genes affecting tumor development. Genes influencing the cancer phenotype that act directly as a result of changes (*e.g.*, mutation) at the DNA level, such as *BRCA1, BRCA2,* and *p53,* are called Class I genes. The Class II genes affect the phenotype by modulation at the expression level. Development of breast cancer and subsequent malignant progression is associated with alterations of a variety of genes of both classes. Many new predictive and prognostic factors have been proposed and studied for breast cancer. HER 2/neu-positive tumors respond poorly to endocrine treatment (31, 32). p53 alteration has an indication of poorer prognosis and poor response to tamoxifen (33, 34). The lack of Nm23 expression has an indicative value of metastatic potential and poor prognosis in invasive ductal carcinoma (35). Cathepsin D, a protease suggested to have a role in breast cancer, appears to affect the potential for invasive growth (11, 14, 36). Positive immunostaining of tumor sections with Factor VIII antibodies seems to be a marker for angiogenesis (37–39). It has been postulated that these tumors are targets for antiangiogenesis drug treatment. Expression of the *mdr-1* gene is proposed to be an indicator of multidrug resistance (38–40). Poor response to endocrine therapy has been indicated for urokinase-type plasminogen activator/plasminogen activator inhibitor-1, a plasminogen activator inhibitor (21). Also receiving major attention are the familial breast cancer-related genes *BRCA1* and *BRCA2* (40–42). With the availability of tens of thousands of EST sequences, we have, using differential cDNA sequence, identified a new putative breast cancer marker gene, *BCSG1*, and studied its expression in breast cancer.

The differential cDNA sequencing method described here is a direct approach that utilizes an automatic EST analysis on a pair of cDNA libraries. Unlike previously described methods, the differential cDNA sequencing approach allows one to identify differentially expressed genes or patterns of genes directly from a computer database. With the advancement of more efficient and rapid sequencing technology, the direct differential cDNA sequencing approach may offer a powerful method for simultaneous analysis of the expression profile of thousands of genes, as well as for the discovery of novel genes of clinical interest.

Using *in situ* hybridization analysis, we have demonstrated the expression of BCSG1 transcripts in the neoplastic epithelial cells of infiltrating breast carcinoma but not in epithelial cells of normal and benign breast. The overexpression (17 of 20) of BCSG1 in malignant infiltrating breast epithelial cells compared to the partial expression (7 of 15) in *in situ* carcinoma suggests that up-regulation of BCSG1 expression is associated with breast cancer malignant progression and may signal the more advanced invasive/metastatic phenotype of human breast cancer. This implication is supported further by the detection of BCSG1 expression in six of eight aggressive Comedo-type DCISs and in only one of seven non-Comedo type DCISs. It is unlikely that BCSG1 is overexpressed as a secondary effect of cellular proliferation, because no detectable BCSG1 expression is evident in rapidly proliferating nonmalignant breast lesions (Fig. 4*F*).

It will be interesting to investigate whether BCSG1 expression in DCIS may indicate a malignant progression leading to invasion and metastasis. There is cause for concern about the large number of DCIS cases that are being diagnosed as a consequence of screening mammography, most of which are treated by some form of surgery. In addition, the proportion of cases treated by mastectomy may be inappropriately high (30). DCIS by definition has intact basement membrane by light microscopy (43). Defective basement membranes, however, have been found when they are stained with periodic acid-Schiff reagent and when they are examined by electron microscopy (44). In fact, it has been reported that re-evaluation by experienced pathologists showed that 28 and 15% of previously diagnosed DCISs demonstrated invasion (45, 46). If BCSG1 expression can provide some prognostic information on distinguishing the DCIS that is not likely to become invasive from the DCIS that is most likely to become invasive, this will help to direct the treatment strategies and to reduce some inappropriate or unnecessary mastectomies.

It is interesting to note that the predicted amino acid sequence of *BCSG1* gene shares a high sequence homology with the non-Aβ component of the AD amyloid precursor protein (28). A neuropathological hallmark of AD is a widespread amyloid deposition resulting from β-amyloid precursor proteins. β-Amyloid precursor proteins are large, membrane-spanning proteins that either give rise to the β-A4 peptide (Aβ fragment; Ref. 47) or a non-Aβ component of AD amyloid (28) that is either deposited in AD amyloid plaques or yielding soluble forms. Although the insoluble membrane-bound AD amyloid destabilizes calcium homeostasis and thus renders cell vulnerable to excitotoxic conditions of calcium influx resulting from energy deprivation or overexcitation (48), the soluble AD amyloid proteins are neuroprotective against glucose deprivation and glutamate toxicity, perhaps through their ability to lower the intraneuronal calcium concentration (49). We currently do not know whether BCSG1 is an instigator or a by-product during breast cancer progression. With the availability of anti-BCSG1 antibody to localize BCSG1 protein and the recombinant BCSG1 protein, we may start to speculate that BCSG1, like soluble AD amyloid, may be potentially involved in protection from tissue damage resulting from tissue remodeling due to the local cancer invasion. An elucidation of the reasons for BCSG1 overexpression in infiltrating breast cancer cells may shed some light on the pathogenesis of breast cancer progression. Nevertheless, we demonstrated a stage-specific BCSG1 expression and an association of BCSG1 overexpression with clinical aggressiveness of breast cancers. The notion that the BCSG1 overexpression may indicate breast cancer malignant progression from benign breast or *in situ* carcinoma to the highly infiltrating carcinoma warrants further investigation.

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Title

Transcriptional suppression of the breast cancer-specific gene (*BCSG1*) expression by the growth inhibitory cytokine oncostatin M

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Running Title

Transcriptional suppression of *BCSG1* by oncostatin M

Key Words

Oncostatin M, Breast cancer progression, Breast cancer specific gene (*BCSG1*), Transcriptional regulation, Cell growth

FOOTNOTES:

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3. The abbreviations used are:

BCSG1	=	breast cancer specific gene 1
CNTF	=	ciliary neurotrophic factor
CT-1	=	cardiotrophin-1
EC_{50}	=	50% effective concentration
EGF	=	epidermal growth factor
FBS	=	fetal bovine serum
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
IL-6	=	interleukin 6
IL-11	=	interleukin 11
IMDM	=	Iscoves modified Dulbecco's medium
LIF	=	leukemia inhibitory factor
OM	=	oncostatin M

ABSTRACT

Recently, a novel breast cancer specific gene, designated *BCSG1*, was isolated from a human breast cancer cDNA library by the differential cDNA sequencing approach. The mRNA of *BCSG1* is highly expressed in the infiltrating carcinomas but not expressed in normal or benign breast tissues. We have detected the mRNA of *BCSG1* in several breast cancer cell lines with the highest expression found in H3922, a cell line derived from an infiltrating ductal carcinoma. Since oncostatin M (OM) has an inhibitory and differentiative effect on H3922 cell growth, we examined the effect of OM on *BCSG1* gene expression and found a dramatic suppression of *BCSG1* mRNA by OM. A kinetic study showed that treatment of H3922 cells with OM initiated an immediate decrease of *BCSG1* mRNA as early as 30 min. By 4 h, the level of *BCSG1* mRNA was decreased to 70% of control, and by 24 h, the mRNA was completely undetectable. The effect of OM on *BCSG1* was concentration-dependent with maximal suppression at concentration of 1 ng/ml. Results from nuclear run-on analysis and mRNA stability studies indicated that the observed OM-induced down-regulation of *BCSG1* mRNA occurs at the transcriptional level. OM treatment of H3922 cells for 16 hours reduced the abundance of actively transcribed *BCSG1* mRNAs to 28.5% of that observed in control cells. These data were further supported by the finding that OM did not affect the stability of *BCSG1* mRNA in actinomycin D-treated H3922 cells. The effect of OM on BCSG1 gene expression was not shared by other OM related cytokines including IL-6, IL-11, and LIF. These cytokines neither inhibited H3922 cell growth nor BCSG1 transcription. Our study suggests that down regulation of BCSG1 expression may be involved in OM-mediated growth inhibition of breast cancer cells.

INTRODUCTION

Breast cancer development and progression is accompanied by multiple genetic changes that lead to qualitative and quantitative alterations in individual gene expression. Consequently, the altered levels of these gene products and their cellular functions will disturb the normal physiological homeostasis of the cells and result in cancer formation. Identification genes that are overexpressed or underexpressed in tumors and subsequent evaluation of their biological functions will help to understand the process of malignant transformation. By utilizing a high-throughput direct-differential cDNA sequencing approach, a novel breast cancer specific gene designated *BCSG1,* was recently isolated from a breast tumor cDNA library (Ji *et al.*, 1997). The *BCSG1* gene is transcripted into a 1 kb mRNA, and the open reading frame of the full length gene is predicted to encode a 127-amino acid polypeptide. Comparison of the predicted amino acid sequence with genetic data base reveals that *BCSG1* is highly homologous to the non-Aβ fragment of human AD amyloid protein with 54% sequence identity (Ueda *et al.*, 1993).

BCSG1 mRNA expression was found exclusively in neoplastic epithelial cells. In situ hybridization analysis has demonstrated a stage-specific expression pattern of *BCSG1* mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade in situ breast carcinoma to high expression in advanced infiltrating carcinomas. This implies that *BCSG1* may play a role in breast cancer malignant progression. This implication is supported by analysis of *BCSG1* mRNA in several breast cancer cell lines. Northern blot detected a 1 kb transcript corresponding to *BCSG1* in 2/4 human breast cancer cell lines derived from pleural effusions and 4/4 breast cancer cell lines derived from ductal infiltrating carcinomas (Ji *et al.*, 1997).

The high level expression of BCSG1 in the neoplastic breast epithelial cells suggest that the expression of BCSG1 may be up-regulated in the mammary gland during the onset and progression of breast cancer. We initiated a study to investigate whether the gene expression of *BCSG1* can be regulated by factors that affect the growth and differentiation of breast cancer cells. The information obtained from such studies may help elucidate the biological functions of *BCSG1*. Previously we demonstrated that H3922 cells, a breast cancer cell line derived from a ductal infiltrating carcinoma, express high level of *BCSG1* mRNA, and that the cellular proliferation of

H3922 cells was inhibited by cytokine oncostatin M (OM) (Liu *et al.*, 1997; Spence *et al.*, 1997).

OM is a 28 kDa glycoprotein produced by activated T lymphocytes and monocytes (Zarling *et al.*, 1986; Brown *et al.*, 1987; Grove *et al.*, 1991). OM is a member of the interleukin-6 (IL-6) family of cytokines, which includes IL-6, interleukin-11 (IL-11), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (Rose *et al.*, 1991; Bazan *et al.*, 1991; Stahl *et al.*, 1993; Zhang *et al.*, 1994). As a pleiotropic cytokine, OM elicits many different biological functions in different cell types. Notable among those is its ability to regulate cell growth and differentiation. OM stimulates the growth of normal fibroblasts (Horn *et al.*, 1990), normal rabbit vascular smooth muscle cells (Grove *et al.*, 1993), human myeloma cells (Zhang *et al.*, 1994), and AIDS-related Kapossi sarcoma cells (Miles *et al.*, 1992; Nair *et al.*,1992). OM also has been shown to inhibit the proliferation of a number of cell lines derived from human tumors including melanoma, breast carcinoma, and lung carcinoma (Horn *et al.*, 1990). In addition to regulating cell growth, OM has been shown to modulate other cellular functions such as induction of plasminogen activator expression in vascular endothelial cells (Brown *et al.*, 1990) and fibroblasts (Hamilton *et al.*, 1991), activation of the acute phase protein gene expression in hepatocytes (Richard *et al.*, 1992), stimulation of metalloproteinase inhibitor TIMP-1 expression in fibroblasts (Bugno *et al.*, 1995), and upregulation of the LDL receptor expression in HepG2 cells (Grove *et al.*, 1991; Liu *et al.*, 1994).

In this study, we have examined the effect of OM on *BCSG1* expression and demonstrated that the *BCSG1* expression is transcriptionally suppressed by OM.

RESULTS

Concentration- and time-dependence of OM-induced suppression of *BCSG1* mRNA expression in breast cancer cells.

Previously, we had detected *BCSG1* mRNA expression in 4 breast cancer cell lines derived from ductal infiltrating carcinoma (ZR-75-1, MDA-MB-231, H3914, and H3922) (Ji *et al.*, 1997). Among these cell lines, H3922 expressed the highest level of *BCSG1* mRNA. Since OM has an inhibitory and differentiative effect on H3922 cell growth, we examined the effect of OM on *BCSG1* mRNA expression. The results in Figure 1 demonstrated a dramatic time-dependent suppression of *BCSG1* mRNA level by OM. Treatment of H3922 cells with OM initiated an immediate decrease of *BCSG1* mRNA as early as 30 minutes. By 4 h treatment, the level of *BCSG1* mRNA was decreased to 70% of that in control, and by 24 h the mRNA was completely undetectable (Figure 1).

We next investigated the concentration-dependent effect of OM on *BCSG1* gene expression. In order to be able to detect *BCSG1* mRNA in OM treated cells, H3922 cells were treated with OM for 6 h at different concentrations before total RNA was harvested for northern blot analysis of *BCSG1* mRNA (Figure 2A). Densitometry analysis of the *BCSG1* hybridization signals with normalization to *GADPH* signals (Figure 2B) showed that cells treated with 0.2, 1.0, 5.0 , 25, and 125 ng/ml OM expressed 58%, 46%, 47%, 45%, and 41% as much *BCSG1* mRNA, respectively, as that observed in control cells treated with OM dilution buffer (1 mg/ml BSA in PBS). These data suggested that OM produced a maximum suppressive effect on *BCSG1* expression at a concentration of 1-5 ng/ml with an EC_{50} of 0.08 ng/ml. To compare the effective dose range for the ability of OM to elicit two different biological responses (cell growth and BCSG1 gene expression), in a parallel experiment the OM-concentration dependent effect on H3922 cell proliferation was also examined. As shown in Figure 2C, the maximal inhibitory effect of OM on H3922 DNA synthesis occurred at OM concentration of 20 ng/ ml with an EC_{50} of 0.2-0.4 ng/ml.

Transcriptional regulation of *BCSG1* expression by oncostatin M.

To determine whether the down regulation of *BCSG1* expression by OM occurs at the transcriptional or post-transcriptional level, we conducted nuclear run-on assays to measure the relative transcription rate of *BCSG1* in control cells and in the cells treated with OM. As shown in

Figure 3, upon the pretreatment with OM for 16 h, H3922 cells contained only 28.5% as many active *BCSG1* mRNA transcripts as observed in control cells. Data were normalized by the signals observed in the *GADPH* slots. The level of reduction of *BCSG1* transcripts is consistent with the results obtained from northern blot analysis. These results suggest that transcriptional regulation is the major component of the observed OM-mediated suppression of *BCSG1* gene expression.

To further investigate the mechanisms by which OM inhibits the expression of the *BCSG1* gene in H3922 cells, *BCSG1* mRNA stability was examined. Control cells and the cells treated with OM for 6 h were exposed to actinomycin D. Total RNAs isolated from the cells at various actinomycin D exposure time points were subjected to northern blot analyses of *BCSG1*. (Figure 4). Although OM treatment reduced the level of *BCSG1* mRNA to approximately 50% of that in control cells, the *BCSG1* mRNA levels in both the control cells and the OM-treated cells were not decreased by actinomycin D. In contrast, the *c-Myc* mRNA levels were rapidly reduced by treatment with actinomycin D. These data suggest that the *BCSG1* mRNA stability was not altered by OM, and that the *BCSG1* mRNA is relatively stable. An attempt to treat cells with actinomycin D for a longer period of time was not successful due to the actinomycin D-mediated toxicity in H3922 cells. These results together with the data generated from the nuclear run-on assay suggest that *BCSG1* gene expression was suppressed by OM at the transcriptional level.

***BCSG1* gene expression was not down regulated by OM-related cytokines.**

Previous studies conducted in our laboratory have shown that the growth-inhibitory activity of OM in the H3922 breast cancer cells is a unique function of this cytokine. Several OM-related cytokines including IL-6, IL-11, and LIF did not inhibit the cellular proliferation of H3922 cells or other breast cancer cell lines such as MCF-7 (Liu *et al.*, 1997). To investigate whether these factors regulate BCSG1 gene expression. H3922 cells were treated with OM, IL-6, IL-11, and LIF for 24 h at a concentration of 100 ng/ml for each factor respectively. The results of northern blot analysis show that BCSG1 mRNA expression was specifically regulated by OM, but not by the other cytokines. These data suggest that the downregulation of BCSG1 expression is concurrently associated with the suppression of cell growth.

DISCUSSION

*BCSG1*was initially identified as a breast cancer specific gene which was expressed in high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library. *In situ* hybridization analysis demonstrated a stage-specific BCSG1 expression: undetectable in normal or benign breast lesions, partial expressed in ductal carcinoma *in situ*, but extremely high level in advanced infiltrating breast cancer (Ji *et al.*, 1997). Overexpression of *BCSG1* in advanced breast carcinomas suggest that *BCSG1* expression could be regulated by factors which play roles in the complex regulation of the growth and progression of breast tumors. In this study, we have shown that this newly identified breast cancer specific gene, *BCSG1* is transcriptionally suppressed by the growth-inhibitory cytokine oncostatin M.

One characteristic of the host response to tumor growth is the infiltration of tumors by macrophages and T lymphocytes. Production of tumor-inhibitory cytokines in a timely and locally (*in situ*) released fashion may represent an important function of the host defense system to suppress tumor progression. From this prospective view, the inhibition of breast tumor growth by OM, a cytokine predominantly produced by activated T cells and macrophages may represent the tumor-host interaction. Currently, the mechanism(s) underlying OM-induced growth inhibition is unknown. It will be interesting to investigate if the OM-induced growth inhibition is mediated in part by down-regulation of *BCSG1* gene that may be involved in facilitating breast cancer progression.

In this study, we show that *BCSG1* gene expression in breast cancer cells is rapidly downregulated by OM. After 24 h treatment with OM, *BCSG1* mRNA was undetectable. Interestingly, the kinetics of OM induced downregulation of *BCSG1* are different than the kinetics of OM-induced downregulation of the *c-Myc* gene in H3922 cells. *C-Myc* mRNA was transiently induced by OM within 1 to 4 h and subsequently suppressed at later times such that maximal suppression (20-30% of control) occurred after 2 to 3 days of culturing cells in the presence of OM (Spence *et al.*, 1997).

The effect of OM on *BCSG1* expression is concentration dependent. The maximal effect occurred at OM concentrations of 1-5 ng/ml with an EC_{50} of 0.08 ng/ml. In contrast, the maximal effective OM concentration for inhibiting H3922 cell growth is 20 ng/ml with an EC_{50} of 0.2-0.4 ng/ml. Comparison of the EC_{50} for the two biological responses presented in this study indicates that

H3922 cells are slightly more sensitive to OM with respect to suppression of *BCSG1* expression than to inhibition of cell growth. It is important to note that cell growth in general is regulated by multiple factors, which affect different levels in a variety of signal transduction pathways. It is possible that downregulation of BCSG1 gene expression by OM is a part of the process leading to growth inhibition of H3922 cells. The higher concentration requirement for OM to inhibit H3922 cell growth than to suppress BCSG1 gene expression suggests that other OM-regulated genes such as *c-Myc* also contribute to the growth inhibitory effect of OM.

The nuclear run-on assays showed that 16 h treatment of cells with OM reduced the abundance of the active transcript *BCSG1* to 28.5% of that present in control cells. This degree of suppression at the transcription level is similar to the extent of suppression of the *BCSG1* mRNA as analyzed by northern blot, indicating that regulation of *BCSG1* gene by OM occurs at the transcriptional level. These data are consistent with the result obtained from the study of *BCSG1* mRNA stability. Treatment of cells with actinomycin D up to 4 h did not decrease the *BCSG1* mRNA level in both control cells and in OM treated cells. In order to determine the half-life of the *BCSG1* mRNA, longer treatment of cells with actinomycin D is required. However, the longer treatment of actinomycin D resulted in cell morphologic changes that were followed by cell death.

It is very interesting to note that in several cell types OM and its related cytokines induce the same biological activities. For example, in HepG2 cells OM, IL-6, and LIF all induce acute phase protein production (Richard *et al.*,1992). In M1 leukemia cells, these cytokines initiate a cell differentiation process (Bruce *et al.*, 1992; Liu *et al.*, 1992). In fibroblasts, TIMP-1 gene expression is induced by OM and LIF (Bugno *et al.*, 1995). However, in breast cancer cells the growth inhibitory activity and suppression of BCSG1 gene expression appears to be OM-specific.

In summary, we have shown that the newly identified *BCSG1* gene is transcriptional suppressed by oncostatin M, a cytokine in the IL-6 family in a concentration-dependent, and time-dependent manner. The results presented in this study suggest that OM-mediated downregulation of the *BCSG1* gene may be involved in growth regression of breast cancer cells. Further investigation of the function of *BCSG1* gene product and its regulation by tumor suppressive agents such as OM will provide important new information for a better understanding of breast cancer development and progression.

MATERIALS AND METHODS

Cells and Reagents. The human breast cancer cell line H3922 was developed from a ductal infiltrating breast carcinoma at the Bristol-Myers Squibb Pharmaceutical Research Institute-Seattle. Cells were cultured in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human recombinant OM was expressed by Chinese hamster ovary cells and purified as previously described (Malik *et al.*, 1992). The other growth factors and cytokines were obtained from R&D Systems, Minneapolis, MN. The plasmid containing the cDNA probe for *c-Myc* was obtained from American Type Culture Collection (Bethesda, MD). A human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) cDNA probe was generously provided by Dr. Jeff L. Ellsworth (CV Therapeutics, Palo Alto, CA).

Northern Blot Analysis. Total cellular RNA was isolated by the method of Peppel and Baglioni (Peppel *et al.*, 1990). Approximately 20 μg of each total RNA sample was separated on a 1.0% formaldehyde agarose gel. RNA was capillary transferred to a Hybond N membrane before crosslinking to the membrane. Prehybridization and hybridization steps were performed under the conditions previously described. The blot was hybridized at 60° C to a 0.55 Kb ^{32}P-labeled human *BCSG1* cDNA probe. The probe was labeled using 50 μCi [α-^{32}P] dCTP with random primed DNA labeling kit (Boehringer Mannheim Corp, Indianapolis, IN). The membrane was then washed 3 times at ambient temperature with 2X SSC, 0.1% SDS and twice at 37° C with 0.1X SSC, 0.1% SDS. The membrane was then dried and exposed to X-OMAT scientific imaging film (Kodak, Rochester, NY) for 1-3 days at -80° C. The *c-Myc* and *GAPDH* probes were prepared by random-primer labeling as described for the *BCSG1* probe. All other steps in analysis of the membrane were also followed as described for the *BCSG1* probe. The autoradiographs were scanned by a laser densitometer (Personal Densitometer™ SI, Molecular Dynamics, Sunnyvale, CA) and the integrated intensity of each band was analyzed with the program ImageQuaNT™, version 4.1. Densitometric analysis of autoradiographs in these studies as well as those discussed below included various exposure times to ensure linearity of signals.

Nuclear Run-on Analysis. These analyses were conducted using a procedure adapted from one that had already been described (Spence *et al.*, 1997). Briefly, 1.8 x 10^7 adherent H3922 cells were harvested with cell scrapers into a minimal volume of cold phosphate buffered saline (PBS). The cells were pelleted by low-speed centrifugation and lysed with lysis buffer (10 mM Tris-HCl, pH

7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The nuclei were pelleted by centrifugation and the lysis procedure was repeated once. The nuclei were recovered by centrifugation a second time and resuspended at 10^8 nuclei/ml in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). The nuclei samples were immediately frozen under liquid nitrogen and stored at -80°C.

The frozen nuclei were subsequently thawed and 100 μl of each sample received 100 μl 2X reaction buffer (70% glycerol, 0.02 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.16 M KCl, 2 mM DTT, 0.2 mM EDTA, 2 mM rATP, 2 mM rCTP, 2 mM rGTP, 2.6 μCi/μl [^{32}P] rUTP). The reactions were incubated with shaking at 30°C for 30 minutes. Labeled nuclei were pelleted and resuspended with 100 μl DNase buffer (50% glycerol, 20 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 10 mg/ml RNase-free DNase I). The reactions were incubated with shaking at 30°C for 15 minutes. Samples were brought up to 125 μl with 7.5 μl 13.6 mg/ml proteinase K, 5 μl 10 mg/ml yeast tRNA, and 12.5 μl 10X SET buffer (5% SDS, 0.05 M EDTA, 0.01 M Tris-HCl, pH 7.4) and incubated at 42°C for 30 minutes.

Labeled RNA transcripts were extracted by adding the following: 275 μl GCSM solution [4 M guanidinium isothiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M b-mercaptoethanol], 45 μl 2.0 M sodium acetate, 450 μl water-saturated phenol, and 90 μl chloroform:isoamyl alcohol (49:1). The samples were vortexed and incubated on ice for 15 minutes. Nuclear run-on transcripts were precipitated with isopropanol and pelleted by high speed centrifugation. Extractions and isopropanol precipitations were repeated and the samples were dissolved with 102 μl TES buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS). Assays for radioactivity were conducted by liquid scintillation. Approximately 2.0 x 10^6 cpm of each nuclear run-on reaction was used as a probe to hybridize a Hybond N membrane (Amersham Life Sciences, Arlington Heights, IL) slot blot. Each blot received the following three plasmids: 5 μg plasmid with the human *GAPDH* cDNA insert, 3 μg of the 0.3 kb fragment of the *BCSG1* cDNA which is the 3' end of the cDNA. This fragment was generated by cutting the *BCSG1* cDNA with the restriction endonuclease BstX1. Probing the *GAPDH* plasmid allowed normalization of the *BCSG1* signals measured by densitometry.

Actinomycin D/mRNA Stability Analysis. H3922 cells in 100 mm tissue culture plates were incubated with or without OM for 6 h. Actinomycin D (5 μg/ml) was added to cells for different

lengths of time. At the end of each time point, total RNA was harvested as described above under "Northern Blot Analysis". Electrophoresis of total RNA samples, blotting, and hybridization to radiolabeled probes were also carried out as described above.

Cell Proliferation Assay. Cells were seeded in 96-well tissue culture plates (Costar, Cambridge, MA) in IMDM medium containing 2% FBS at a density of 3000 cells/well in 100 μl of medium. Three to 5 hours after seeding, 50 μl of the same culture media containing various factors was added to each well. Three days later [^{3}H]thymidine (0.5 μCi/50 μl/well) in medium was added to the culture plates 4 hours prior to harvest. The amount of [^{3}H]thymidine incorporated into cells was measured using a liquid scintillation counter (Pharmacia, Piscataway, NJ). The differences in counts/min incorporated between experimental and control cultures were used as an index for DNA synthesis. Each data point represents the average of triplicate cultures and each experiment was performed at least 3 times.

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FIGURE LEGENDS

Figure 1. Time-dependent suppression of *BCSG1* mRNA expression by OM. Total RNA (20 μg/lane) was isolated from H3922 cells that were cultured in 2% FBS IMEM and treated with OM at a dose of 20 ng/ml for the indicated lengths of time. RNA samples were blotted onto a nylon membrane and hybridized to a ^{32}P-labeled 0.55 Kb *BCSG1* cDNA probe as described in "Materials and Methods". The blot was rehybridized under the same conditions with a ^{32}P-labeled human *GAPDH* probe. Radioactive signals were detected by autoradiography and quantified by densitometry.

Figure 2. OM concentration-dependent effects on *BCSG1* mRNA expression and on cellular proliferation.

A. Total RNA (20 μg/lane) was isolated from H3922 cells that were untreated (lane 1), 0.2 ng/ml OM-treated (lane 2), 1.0 ng/ml OM-treated (lane 3), 5.0 ng/ml OM-treated (lane 4), 25 ng/ml OM-treated (lane 5), or 125.0 ng/ml OM-treated (lane 6). All OM treatments were for 6 h. RNA samples were analyzed as described in Figure 1. **B**. The relative levels of *BCSG1* mRNA normalized to *GADPH* mRNA on the autoradiographam shown in panel A were quantified by scanning densitometry. The results shown are representative of two separate experiments. **C**. H3922 cells (3000 cells/well) were incubated for 72 hours in IMDM containing 2% FBS with the indicated amount of purified human recombinant OM. Cells were pulsed with [^{3}H]thymidine for an additional 4 hours. The amount of radioactivity incorporated into the cells was determined by scintillation assay and the data were expressed as percentages of that observed in the untreated controls.

Figure 3. Nuclear runon analysis of *BCSG1* transcription. Two slots were blotted onto each of two nylon membrane strips. One slot received 3 μg of the 0.3 kb fragment of the *BCSG1* cDNA, which is the 3' end of the cDNA. This fragment was generated by cutting the *BCSG1* cDNA with the restriction endonuclease BstX1. The second slot was loaded with 5 μg of the *GAPDH* plasmid. One nylon strip was hybridized to a ^{32}P-radiolabeled nuclear runon reaction prepared from 16-h OM-treated H3922 cells. The second was hybridized to a labeled nuclear runon reaction prepared from

control cells. Equal amounts of radioactivity were used in each hybridization. Radioactive signals were detected by autoradiography and quantified by densitometric analysis. Isolation of nuclei, preparation of nuclear run-on reactions, hybridizations, and washes were all as described in "Materials and Methods".

Figure 4. OM did not change *BCSG1* mRNA stability. Cells were either treated with OM at the concentration of 50 ng/ml for 6 h (lane 7-12), or treated with OM dilution buffer, 1 mg/ml BSA in PBS, for the same length of time (lane 1-6), then actinomycin D at a concentration of 5 μg/ml was added to cells for different lengths of time. At the end of each time point, total RNA was harvested and analyzed for *BCSG1* mRNA and *c-Myc* mRNA expressions as described in Figure 1. The length of actinomycin D treatment: lanes 1 and 7, 0 h; lanes 2 and 8, 0.5 h; lanes 3 and 9, 1 h; lanes 4 and 10, 2 h; lanes 5 and 11, 3 h; lanes 6 and 12, 4 h.

Figure 5. Comparison of effects of OM related cytokines on *BCSG1* gene transcription. H3922 cells were treated for 24 h with each factors at 100 ng/ml concentration respectively. Total RNA was subsequently isolated for northern blot analysis of *BCSG1* mRNA levels.

Fig. 1

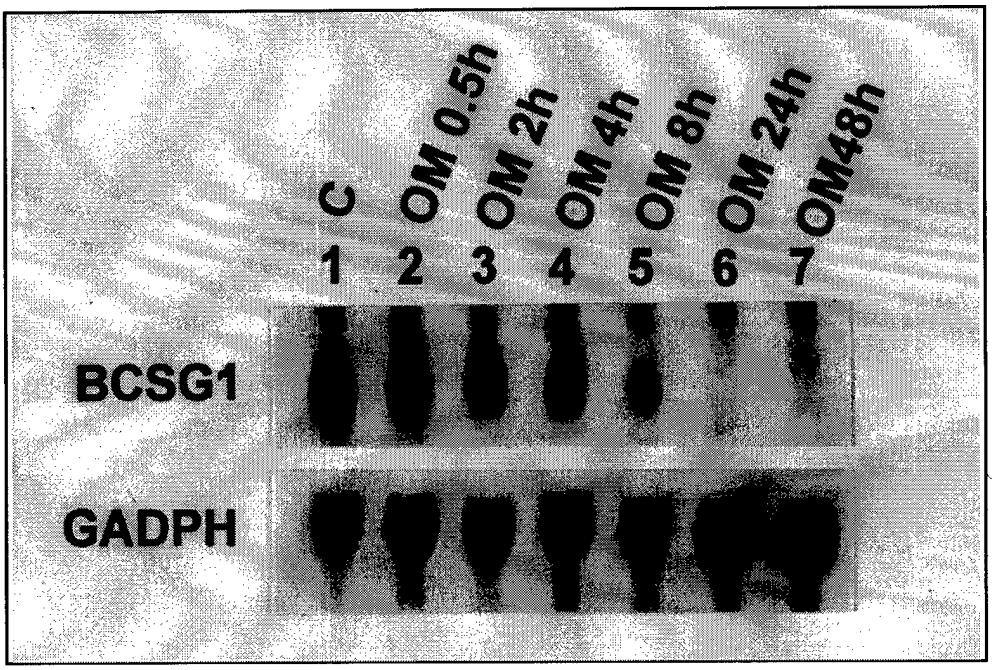
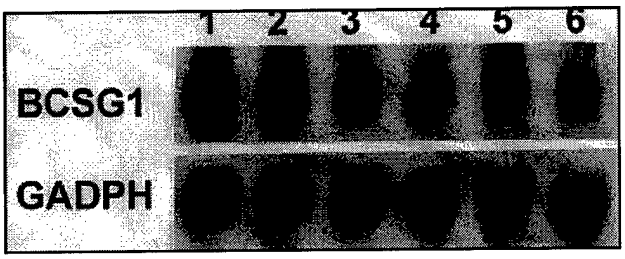


Fig. 2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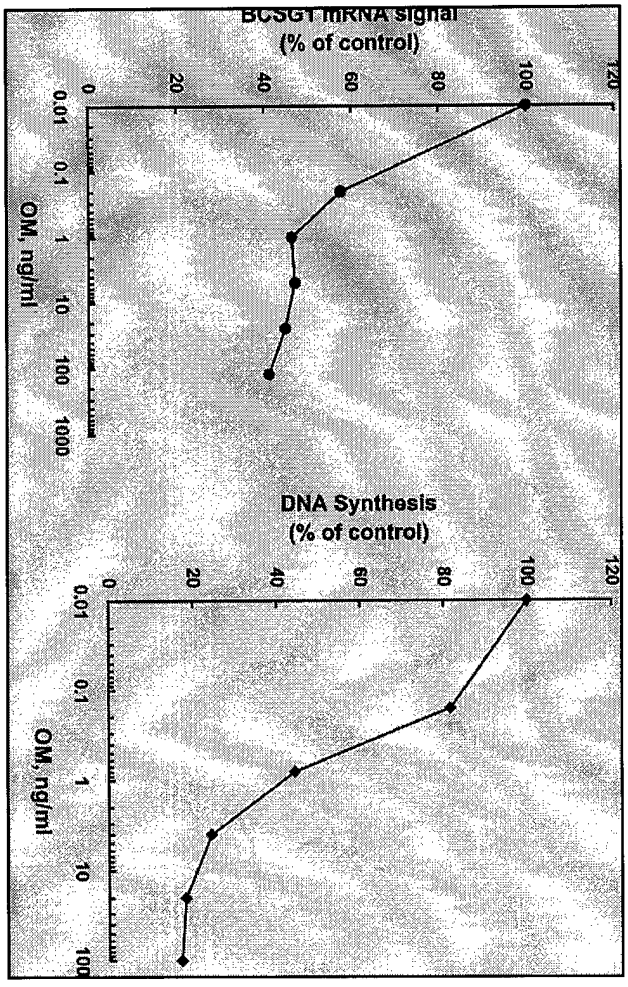
BCSG1
GADPH

A


BCSG1 mRNA signal
(% of control)
0 20 40 60 80 100 120
0 0.01 0.1 1 10 100 1000
OM, ng/ml
DNA Synthesis
(% of control)
0 20 40 60 80 100 120
0 0.01 0.1 1 10 100
OM, ng/ml

B

C

Fig. 3

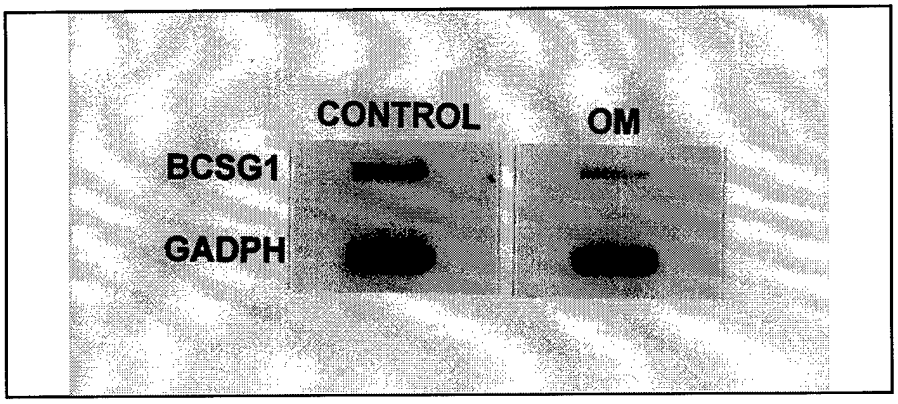


Fig. 4

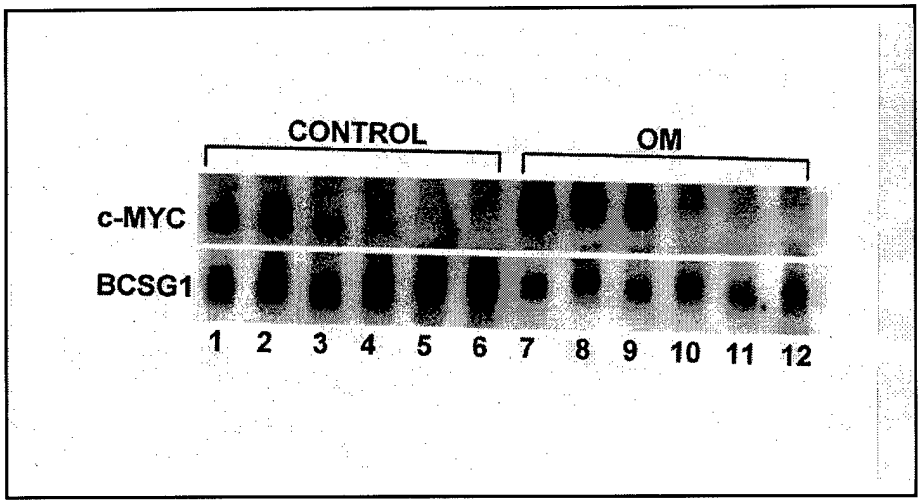


Fig. 5